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Genetic Variation in Neuraminidase Genes of Influenza A (H3N2) Viruses

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Nucleotide sequences of the neuraminidase (NA) genes of 33 influenza A (H3N2) epidemic strains isolated between 1968 and 1995 were analyzed to determine their evolutionary relationships. Phylogenetic analysis using the DNA maximum-likelihood method indicates that the NA genes of recent H3N2 field strains, like their hemagglutinin genes (HA), have evolved as two distinct lineages represented by the vaccine strains, A/Beijing/353/89 and A/Beijing/32/92 (or A/Shanghai/24/90). Furthermore, genetic reassortment of NA genes between the two lineages occurred during their circulation. Genetic reassortants, which bear an A/Beijing/32/92-like HA and an A/Beijing/353/89-like NA, have circulated worldwide and are representative of current influenza A (H3N2) epidemic strains. The mutation rate of the NA gene was found to be 2.28×10^{-3} per nucleotide site per year with 42% of the mutations resulting in amino acid substitutions. Thirty-five percent of the amino acid substitutions was located in sites previously suggested to be reactive to antibody. Amino acid residues involved in NA enzyme activity have been conserved. Seven potential glycosylation sites identified in the NA of A/Hong Kong/8/68 virus were conserved by the majority of isolates, with more recently circulating viruses having an additional glycosylation site. Comparison of the rate of amino acid substitutions in the NA stalk to that of entire NA revealed high variability in this region. These findings demonstrate the importance of closely monitoring both the HA and the NA genes of influenza viruses to aid vaccine strain selection.

INTRODUCTION

Type A influenza viruses are enveloped and have two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The HA performs two crucial roles in the early stage of virus replication. It binds to a sialic acid-containing receptor on the cell surface and, after receptor-mediated endocytosis of the virus particle, causes fusion of the viral envelope and intracellular membranes under acidic conditions leading to the liberation of the genome into the cytoplasm (for reviews see Lamb and Choppin, 1983; Lamb, 1989) The role of NA in replication and maturation of the virus has not been characterized as thoroughly (for reviews see Colman, 1989; Air and Laver, 1989). Early studies suggested that NA may function in the final stages of infection (Kilbourne *et al.,* 1968; Webster and Laver, 1967). Several other studies supported the early findings and suggested that NA prevents formation of virus aggregates and releases newly assembled virus particles from the cell surface by removing sialic acid from the virus envelope proteins (Compans *et al.,* 1969; Palese *et al.,* 1974; Palese and Compans, 1976). NA may also help the virus gain access to the cell by catalyzing the cleavage of the a-ketosidic linkage between the terminal sialic acid and the adjacent sugar residue in mucus secretions (Tabak *et al.,* 1982). A more recent study suggested that influenza A neuraminidase does not play an essential role in viral entry, replication, assembly, or budding (Liu and Air., 1994). As major surface antigens of influenza A viruses, HA and NA both undergo antigenic shift and antigenic drift. Antibody to HA is the most important determinant of immunity because it can neutralize the infectivity of influenza virus (Dowdle *et al.,* 1973; Hobson *et al.,* 1972; Couch and Kasel. 1983). Although anti-NA antibodies do not neutralize virus infectivity (Kilbourne *et al.,* 1968), they appear to modify the disease and reduce both pulmonary virus titer and the extent and severity of lung lesions (Schulman, 1975). Therefore, the immune response to NA also has an important role in the epidemiology of influenza.

Since their emergence during the 1968 pandemic, influenza A (H3N2) viruses have been circulating continuously in humans and have caused significant cumulative morbidity and mortality. For example, severe influenza epidemics and the resultant excess mortality observed during the 1975–76, 1989–90, and 1993–94 influenza seasons were the results of wide spread circulation of A/Victoria/3/75, A/Shanghai/11/87, and A/Beijing/32/92-like variants, respectively (CDC, 1976, 1990, 1994; Both *et al.,* 1983). Molecular epidemiology coupled with disease and virologic surveillance for influenza A (H3N2)

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epidemic strains are important for determining their epidemic impact and for the selection of appropriate vaccine strains. Previous studies on antigenic drift of viruses of the influenza A (H3N2) subtype have focused mainly on variation of HA (Verhoeyen *et al.,* 1980; Both and Sleigh, 1981; Skehel *et al.,* 1983; Both *et al.,* 1983; Nakajima *et al.,* 1988; Bean *et al.,* 1992; Cox *et al.,* 1993; Cox and Bender, 1995). In contrast, much less is known about the evolution of the NA gene (Laver *et al.,* 1982; Martinez *et al.,* 1983; Nakao *et al.,* 1993). In the present study, genetic variation of the NA genes of influenza A (H3N2) epidemic strains isolated world-wide between 1968 and 1995 was examined. Data obtained from this study were compared with those from previous studies in order to increase our understanding of the evolutionary pattern of epidemic influenza A (H3N2) viruses and to assist in vaccine strain selection.

MATERIALS AND METHODS

Viruses

Influenza A (H3N2) viruses examined in this study were propagated in the allantoic cavity of 9- to 11-day-old embryonated hen's eggs. Table 1 lists the H3N2 viruses examined, their abbreviations, the date of collection, and the HA antigenic characteristics determined by hemagglutination inhibition (HI) tests (Kendal *et al.,* 1979). The virus strains were chosen either because they were representative of viruses isolated during a season or particular epidemic or because they were antigenic variants distinguishable in HI tests using postinfection ferret sera. In addition, an attempt was made to obtain a wide geographic distribution of virus strains.

Nucleotide sequencing

Virion RNA that had been extracted as described previously (Palese and Schulman, 1976) was used for PCR amplification(Xu *et al.,* 1993; Rocha *et al.,* 1991). cDNA synthesis and PCR amplifications of the coding region of NA genes were carried out using forward primer 14 (5′ d-GTGAAGATGAATCCAAATCAA) and reverse primer 1420 (5′ d-GCGAAAGCTTATATAGGC). The PCR-derived dsDNA was used as a template for automated sequencing on an Applied Biosystem 373A automated DNA sequencer using cycle sequencing dyeterminator chemistry (Perkin–Elmer, Foster City, CA). Primer 14 (see above) and five internal primers complementary to the viral RNA strand were used to sequence the coding region of the NA gene: 106 (5′ d-GGTAACTACTGTAACATTGCA), 372 (5′ d-GAGAACCTTATGTGTCATGCG), 695 (5′ d-CAGGAGTCGGAATGCGTTTGT), 823 (5′ d-GTCAGGAAGTGCTCAGCA) and 1063 (5′ d-AGTGAAAGGCTGGGCCTT).

The nucleotide sequences were stored and analyzed in a Digital Corporation VAX computer (Minneapolis) using version 8.0 of the sequence analysis software package of the University of Wisconsin at Madison Genetic Computer Group (Devereaux *et al.,* 1984). Version 3.5 of the Phylogeny Inference Package (Phylip; Felsenstein, 1989) was used to estimate phylogenies from the nucleotide sequences. GenBank accession numbers for the sequences obtained from this study are shown in Table 1.

RESULTS

Epidemiologic background

Influenza A (H3N2) viruses were first isolated from humans during the 1968 pandemic. Several important antigenic variants of the H3N2 subtype have appeared since then and have caused regional epidemics or outbreaks in many areas of the world. Some notable variants have been the A/Victoria/3/75-like, A/Bangkok/1/79-like, A/Mississippi/1/85-like, and A/Shanghai/11/87-like strains (Both *et al.,* 1983; WHO, 1986, 1987; Cox *et al.,* 1993; CDC, 1976, 1990). Between 1989 and 1990, two distinct antigenic variants of the influenza A (H3N2) subtype were recognized in hemagglutination-inhibition tests with postinfection ferret serum (CDC, 1993). These viruses were antigenically related to either A/Beijing/353/89 (BE35389), a representative epidemic strain circulating in China, South Asia and the Southern Hemisphere during the 1989–90 influenza season, or A/Shanghai/24/90 (SH2490), a variant isolated in China in August 1990 (CDC, unpublished data). BE35389-like viruses gradually spread to Europe and North America and caused epidemics during the 1991–92 influenza season, while SH2490-like viruses circulated in China and Southern Asia at low levels of activity (CDC, unpublished data). In the early spring of 1992, A/Beijing/32/92 (BE3292)-like strains which were antigenically and genetically related to the SH2490 reference strain were isolated from a local influenza epidemic in northern China (Guo Yuanji, personal communication). BE3292-like variants began to circulate worldwide in humans and became the major epidemic strain (CDC, 1993). Influenza activity caused by this variant resulted in epidemics in the Northern Hemisphere during the 1993–94 influenza season. Phylogenetic studies of the HA1 domain of HA genes of these influenza A (H3N2) viruses revealed that the HA genes of BE35389-like viruses and BE3292 (SH2490)-like viruses are located on two different branches of the evolutionary tree (Fig. 1; HA sequences data will be discussed in a future publication).

Evolution of N2 subtype neuraminidase

Phylogenetic analysis of nucleotide sequences. The nucleotide sequences of the coding region of the NA gene were obtained for 33 strains of influenza A (H3N2)

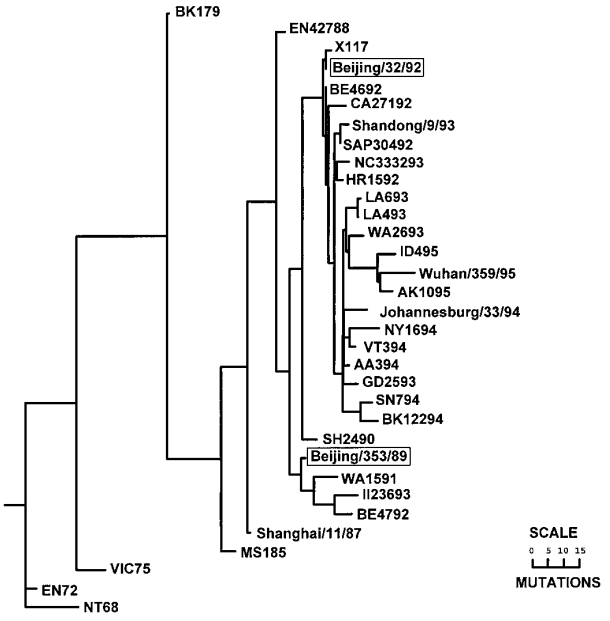

FIG. 1. Evolutionary tree of 33 influenza A (H3N2) virus HA gene sequences rooted to the A/NT/60/68 strain. The sequence data were analyzed by using version 8.0 of the sequence analysis software package of the University of Wisconsin Genetics Computer Group. Version 3.5 of PHYLIP with the DNAML program was used to estimate phylogenies from nucleotide sequences. Horizontal lines are proportional to the numbers of nucleotide differences between branch points; vertical lines are for spacing. Scale is drawn to show the mutational distances between the viruses. Vaccine strains are identified by their full strain designation.

viruses isolated from 1968 to 1995 (Table 1). The single open reading frame of the NA spanned positions 20 through 1429 and coded for a predicted polypeptide of 469 amino acids. Phylogenetic analysis was performed using the Phylogeny Inference Package (PHYLIP version 3.5) with the DNAML (maximum likehood algorithm) program (Felsenstein, 1989). Figure 2 shows the evolutionary tree of the NA genes for 38 nucleotide sequences including 5 from previous studies (Bentley and Brownlee, 1982; Markoff and Lai, 1982; Van Romput *et al.,* 1982; Martinez *et al.,* 1983; Li *et al.,* 1992). The NA genes of viruses isolated from 1968 to 1988 appeared to have shared a single main lineage with short side branches; however, the NA genes of viruses isolated from 1989 to 1992 were divided into two distinct branches represented by the BE35389 and BE3292 (SH2490) strains, respectively. This observation indicated that the NA genes of recent influenza A (H3N2) viruses, like their HA genes (Fig. 1), have evolved as two lineages (Fig. 2). Seventeen strains had NA genes located on the same branch as that of BE35389 (Fig. 2). These strains are shown in lower case letters signifying that their HA genes were closely related to that of the BE3292 strain (Table 1, Fig. 1) although the NA genes of theses viruses were closely related to that of the BE35389. This finding indicates that genetic reassortment of NA genes between the two lineages occurred sometime during their cocirculation.

Analysis of amino acid sequences. Figure 3 shows 38 deduced amino acid sequences for the field strains examined between 1968 and 1995. Inspection of the sequences reveals two highly conserved regions that were unchanged during that 27-year period. One is the N-terminal 12 amino acids, and the other is a 24-amino acid region from residue 267 to 289 (Fig. 3). A 30 amino acid region between residue 95 and 125 is also highly conserved with only one virus (BK179) having a change in this region during 27 years (Fig. 3). There are also two regions with multiple mutations. One is a 43 amino acid region between residues 36 and 78 where 17 substitutions were observed. Another highly variable region where 38 substitutions have occurred is between residues 300 and 430 which is located in the head of NA (Fig. 3).

The NA proteins and genes of BE35389-like viruses differed from the BE3292 (SH2490)-like viruses by 6 amino acids and 9 nucleotides (Fig. 3 and data not shown). Characteristic amino acids of BE35389-like viruses are Asn at position 43, Leu or Gln at position 52, and Arg at position 400. The characteristic amino acids of the BE3292 (SH2490)-like viruses are Ile at position 51, Gln at position 54, and Val at 262 (Fig. 3).

Seven potential glycosylation sites in the NA of HK868 virus were conserved in the 33 subsequent isolates examined except for MS185, which lost the potential glycosylation site at residues 402–404, and HE89 virus, which lost the site at residues 200–202. Twenty-five strains isolated after 1989 acquired an additional potential glycosylation site at residues 329–331, and SAP92 had a second additional site at 309–311 (Fig. 3). Amino acid residues located in the substrate binding pocket (Air and Laver, 1989) have been completely conserved in all strains examined (Fig. 3).

Estimation of evolution rates. The rates of evolution of the NA genes were calculated by using linear regression analysis. Rates were determined not only for the entire NA coding region, but also for the NA stalk region. The nucleotide substitution rate for the entire coding region was 2.28×10^{-3} /site/year (with 42% of the mutations resulting in amino acid substituions), which was similar to that for the stalk (2.58×10^{-3} /site/year). The amino acid substitution rate for the entire NA coding region was 2.56×10^{-3} /site/year. The rate for NA stalk, however, was 4.21×10^{-3} /site/year, which was higher than that of the entire coding region (data not shown).

DISCUSSION

Close monitoring of the evolution of the HA and NA genes of influenza A viruses is important in the selection

TABLE 1

Influenza A (H3N2) Viruses Examined in the Current Study

Name	Abbreviation	Date of collection	HA antigenic[a] characteristics	GenBank Accession No.	Source of reference
1. A/HONG KONG/8/68	HK868	uk,[b] 1968	HK68-like	U42630	This report
2. A/NT/60/68	NT68	uk, 1968	NT68-like		Bentley and Brownlee, 1982
3. A/HONG KONG/107/71	HK10771	uk, 1971	Nd[C]	u42631	This report
4. A/UDORN/72	UDORN72	uk, 1972	UDORN72-like		Lewis and Lai, 1982
5. A/VICTORIA/3/75	VIC75	uk, 1975	VIC75-like		Rompuy *et al.,* 1982
6. A/BANGKOK/1/79	BK179	uk, 1979	BK179		Martinez *et al.,* 1983
7. A/MISSISSIPPI/1/85	MS185	Jan, 1985	MS185-like	U42632	This report
8. A/SHANGHAI/11/87	SH1187	Aug, 1987	SH87-like	U42633	This report
9. A/ENGLAND/427/88	EN42788	Winter, 1988	SH87-like	U42634	This report
10. A/HEBEI/24/89	HE89	March, 1989	N/A		Li *et al.,* 1992
11. A/BEIJING/353/89	BE35389	Nov, 1989	BE35389-like	U42635	This report
12. A/SHANGHAI/24/90	SH2490	June, 1990	SH2490[d]-like	U42636	This report
13. A/WASHINGTON/15/91	WA1591	Mar, 1991	BE35389-like	U42637	This report
14. X-117	X-117	N/A	BE3292-like	U43427	This report
15. A/BEIJING/32/92	BE3292	Feb, 1992	BE3292-like	U42770	This report
16. A/BEIJING/46/92	BE4692	Feb, 1992	BE3292-like	U42771	This report
17. A/BEIJING/47/92	BE4792	Feb, 1992	BE35389-like	U42772	This report
18. A/HARBIN/15/92	HR1592	Dec, 1992	BE3292-like	U42773	This report
19. A/CALIFORNIA/271/92	CA27192	Nov, 1992	BE3292-like	U42774	This report
20. A/NETHERLANDS/938/92	NED93892	uk, 1992	BE3292-like	U42775	This report
21. A/SAPPORO/304/92	SAP30492	Dec, 1992	BE3292-like	U42776	This report
22. A/GUANGDONG/24/93	GD2493	June, 1993	BE3292-like	U42777	This report
23. A/GUANGDONG/25/93	GD2593	June, 1993	BE3292-like	U42778	This report
24. A/NANCHANG/3332/93	NC333293	April, 1993	BE3292-like	U42779	This report
25. A/INDIA/236/93	II23693	Nov, 1993	BE35389-like	U42780	This report
26. A/LOUISIANA/4/93	LA493	Aug, 1993	BE3292-like	U43417	This report
27. A/LOUISIANA/6/93	LA693	Sep, 1993	BE3292-like	U43418	This report
28. A/SHANDONG/9/93	SD993	Oct, 1993	BE3292-like	U43419	This report
29. A/WASHINGTON/26/93	WA2693	uk, 1993	BE3292-like	U43420	This report
30. A/ANN ARBOR/3/93	AA393	uk, 1993	BE3292-like	U43421	This report
31. A/SINGAPORE/7/94	SN794	June, 1994	BE3292-like	U43422	This report
32. A/VERMONT/3/94	VT394	Jan, 1994	BE3292-like	U43423	This report
33. A/NEW YORK/16/94	NY1694	Nov, 1994	BE3292-like	U43424	This report
34. A/JOHANNESBURG/33/94	JHB3394	July, 1994	BE3292-like	U43425	This report
35. A/BANGKOK/122/94	BK12294	June, 1994	BE3292-like	U43426	This report
36. A/ALASKA/10/95	AK1095	Feb, 1995	BE3292-like	U51245	This report
37. A/WUHAN/359/95	WU35995	Sep, 1995	BE3292-like	U51246	This report
38. A/IDAHO/4/95	ID495	Nov, 1995	BE3292-like	U51247	This report

[a] Determined by hemagglutination inhibition tests and/or HA sequencing analysis.
[b] Month of isolation unknown.
[c] Not determined.
[d] The precursor of BE3292.

of appropriate vaccine strains. Neuraminidase inhibition assays, the conventional method for antigenic analysis of the NA, are rather complicated tests for which special reassortant viruses are required. In the current study, we used sequence and phylogenetic analysis to examine the evolution of the NA gene. Our results confirmed that molecular analysis of influenza virus genes is useful for studying virus evolution and emphasized the need to sequence both the HA and the NA genes of field strains in order to achieve as close a match as possible for both surface antigens between the vaccine and circulating strains. For example, SD993 virus was selected to replace the BE3292 virus in the influenza vaccine for the 1994–1995 influenza season (CDC, 1994). It was selected because not only the HA, but also the NA gene of this virus represented the majority of the field isolates circulating at the time (Figs. 1 and 2).

An early study on the evolution of influenza viruses suggested that human influenza A virus evolved along a single lineage without cocirculating strains of viruses of the same subtype but different lineages (Yamashita *et al.,* 1988). Recent studies of the HA genes of influ-

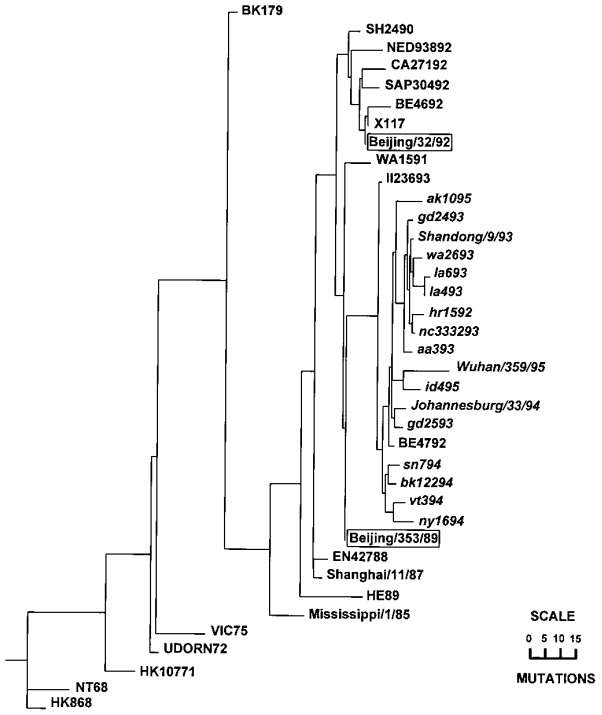

FIG. 2. Evolutionary tree of 38 influenza A (H3N2) virus NA gene sequences rooted to the A/Hongkong/8/68 strain. The sequence data were analyzed by using version 8.0 of the sequence analysis software package of the University of Wisconsin Genetics Computer Group. Version 3.5 of PHYLIP with the DNAML program was used to estimate phylogenies from nucleotide sequences. Horizontal lines are proportional to the numbers of nucleotide differences between branch points, vertical lines are for spacing. Scale is drawn to show the mutational distances between the viruses. Vaccine strains are identified by their full strain designation.

enza A epidemic strains revealed that separate lineages of influenza A (H1N1) and (H3N2) subtypes can cocirculate in humans (Cox *et al.,* 1989, 1993; Cox and Bender, 1995). Nakao and his colleagues have suggested that the NA (and NS) genes of human influenza A (H2N2) strains isolated 1967–1968 could be divided into two groups (Nakao *et al.,* 1993). Our phylogenetic analysis revealed that the NA gene of the BE35389 virus had evolved from the previous SH1187 epidemic strain. The NA gene of the SH2490 reference strain apparently evolved along a separate evolutionary pathway and cocirculated with BE35389-like viruses between 1990 and 1992 (Fig. 2). This indicates that the NA genes of recent H3N2 strains, like their HA genes, have evolved as two distinct lineages. BE35389-like and BE3292 (SH2490)-like viruses cocirculated between 1990 and 1992, thus providing an opportunity for mixed infections that might lead to the emergence of reassortants. The isolation of H3N2 field strains bearing a BE3292-like HA and a BE35389-like NA indicates that reassortment of the NA genes between these two lineages has occurred. Such reassortant viruses have been isolated in several countries (Table 1), making it unlikely that the reassortment occurred during laboratory isolation. These reassortants would not be expected to have any increased epidemic potential compared with their parents because a significant part of the population had already developed immunity to these antigens from previous infection or immunization. Molecular epidemiologic data obtained from this study demonstrated that a BE3292-like virus (CA27192) having both HA and NA from the BE3292 strain was isolated in North America before the detection of reassortants. The CA27192 was isolated 1 month earlier than the first identified reassortant HR1592, which was from China. Since all recent H3N2 strains isolated worldwide have the same genotype as HR1592 (Fig. 2), it is not possible to determine if the reassortants emerged in China first and then spread to other countries or if reassortment occurred more than once in different areas.

Sequence analysis of the entire coding region of the NA genes of the 38 H3N2 field strains revealed that 3,584 of 53,580 (6.68%) nucleotides sequenced have changed and 1,472 of 17,822 (8.25%) amino acids have changed as compared to HK68 virus. 517 of 1472 (35.1%) amino acid changes are located in previously defined antigenic sites on the head of the NA tetramer (Colman *et al.,* 1983). This finding suggests that the antigenicity of the NA of the H3N2 viruses has continued to undergo variation. Examination of the potential glycosylation sites in the deduced amino acid sequences revealed that 25 recent isolates acquired an additional site at position 329–331, which is located in antigenic site 1 (Colman *et al.,* 1983). Because glycosylation of the viral surface glycoproteins plays a role in antigenic variation by masking and unmasking antigenic sites (Raymond *et al.,* 1986; Wilson *et al.,* 1983; Skehel *et al.,* 1984; Rota *et al.,* 1990; Schild *et al.,* 1983, Robertson *et al.,* 1985), this additional glycosylation site may also contribute to antigenic variation in the NA of recent influenza A (H3N2) viruses.

Using a relatively large database of sequences, we have calculated the rates of nucleotide and amino acid substitutions by using linear regression analysis. Mutation rates for the entire coding region of NA calculated in this study (2.28×10^{-3} /site/year) were lower than that reported previously (Martinez *et al.,* 1983). The difference in mutation rate, however, probably reflects differences in the methods used and in the number and epidemiologic significance of the viruses chosen for analysis in each study.

Although the functional role of the stalk region has not been fully established (for review see Air and Laver, 1989), a recent study revealed that the length of the NA stalk can affect the host range of influenza A viruses (Castrucci and Kawaoka, 1993). In another report, amino

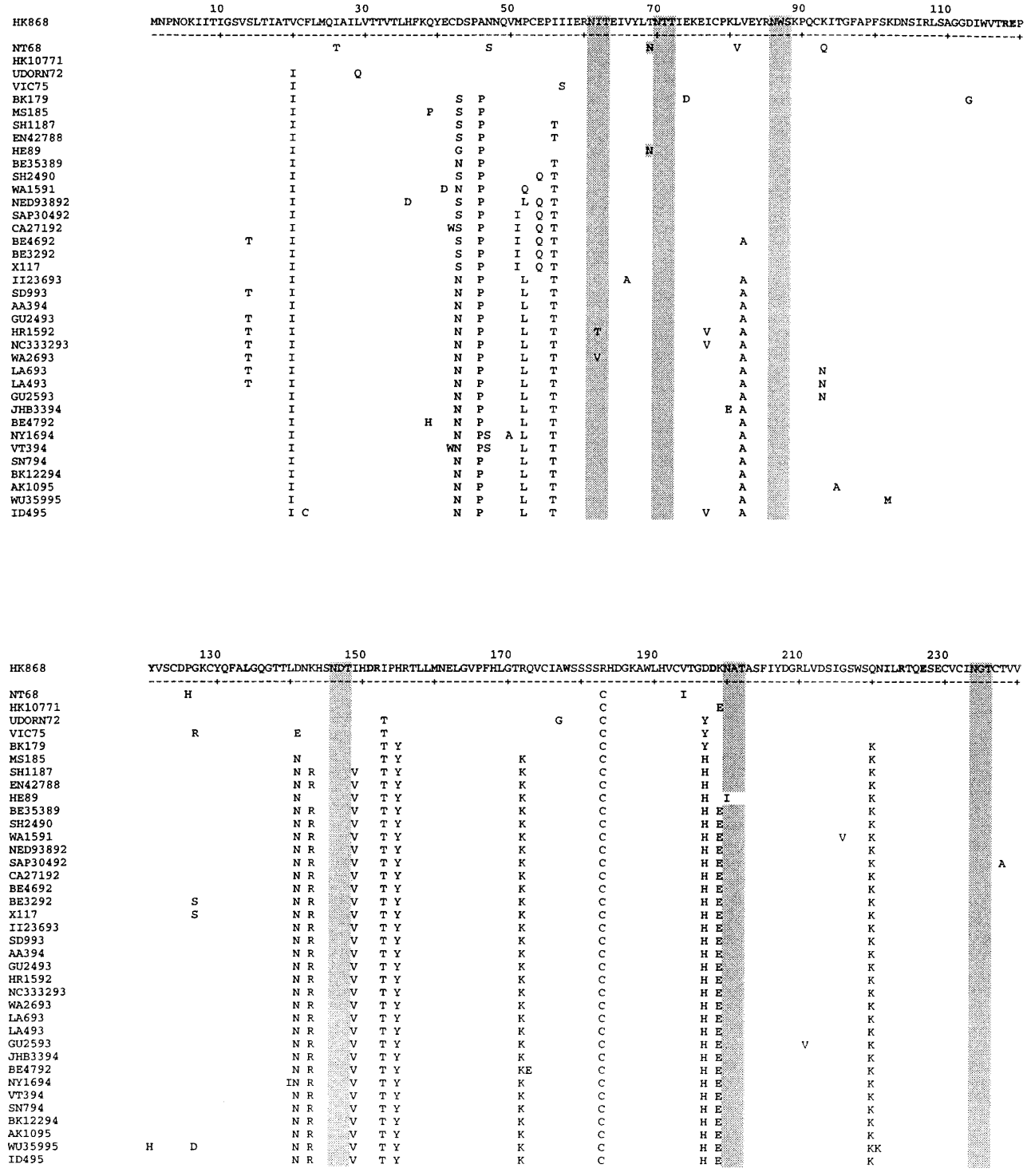

FIG. 3. Deduced amino acid sequence of the NA gene of A/Hong Kong/8/68 compared to the sequences of H3N2 viruses isolated from 1968 to 1994. Differences between HK868 and the other strains are shown. Potential glycosylation sites (N-X-S/T) are shaded. Conserved residues in the substrate binding pocket are shown in boldface.

acid deletions in the stalk resulted in virions which lacked enzyme activity with large substrates, and which were not released from erythrocytes (Els *et al.,* 1985). Blok and Air reported that the sequence of the NA stalk varies considerably among different viruses, even within the same subtype (Blok and Air, 1980, 1982). Our data has revealed that the stalk region of the NA of influenza A (H3N2) viruses is variable (Fig. 3) with the

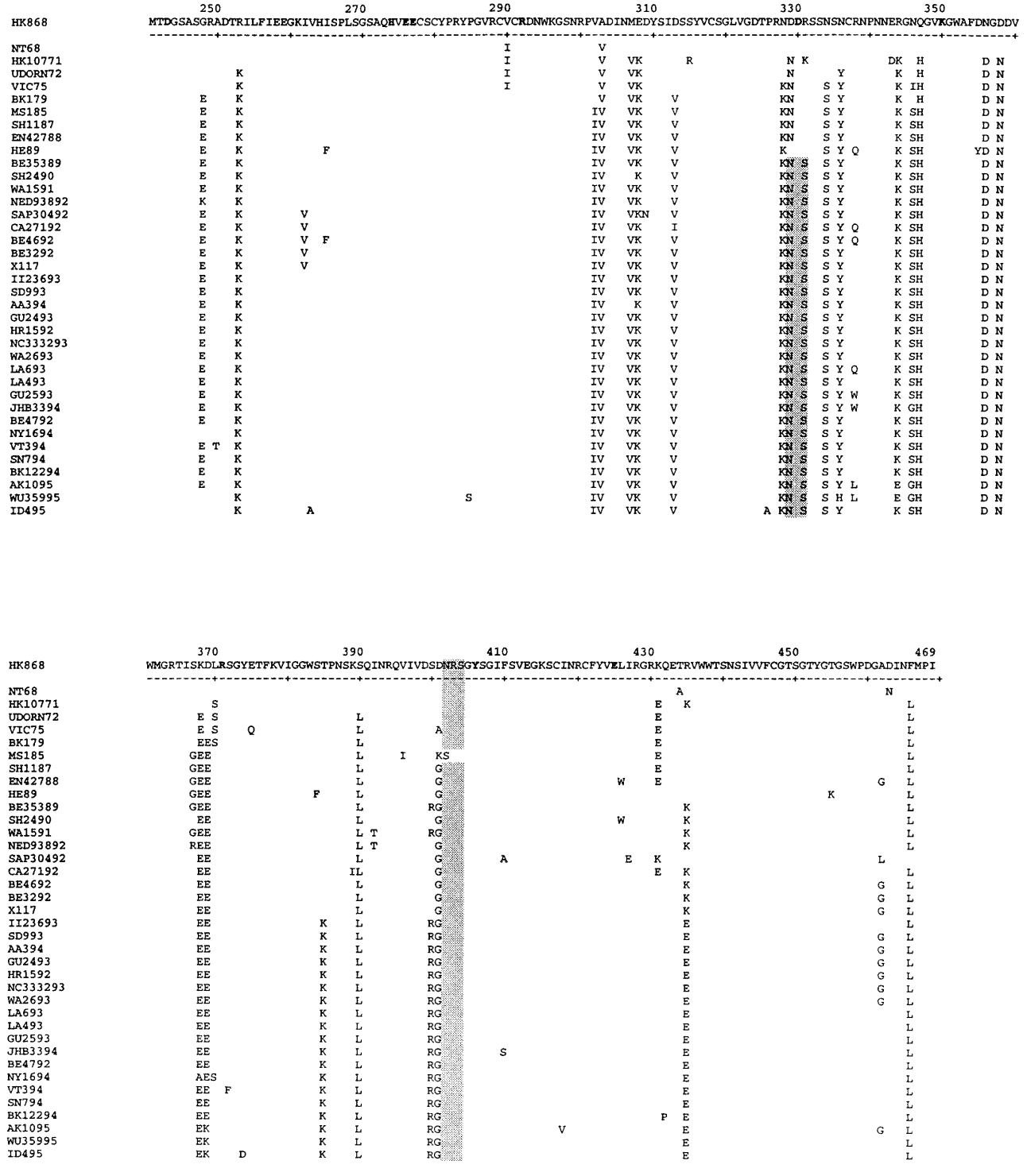

FIG. 3 — *Continued*

rate of amino acid substitution in the stalk being higher than that of the entire coding region or of the head alone. Possible reasons for high variability in this region might include the lack of functional constraints other than length or host immune pressure.

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